Reagent components in this kit require different storage conditions. Upon receipt, store reagents as indicated on the reagent labels.

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Amine Coupling Reagent Kit

Reagent Kit for use with Amine Reactive Biosensors
Product Code: 18-5017

Read the entire product insert fully before beginning the assay.

OVERVIEW

The Amine Coupling Reagent Kit is a set of reagents qualified by Fortebio to allow for the efficient coupling of purified proteins of interest onto the Amine Reactive Biosensor surface.

INTENDED USE

This kit is intended to provide the Octet System user with a convenient source for all the reagents needed to immobilize a protein of interest onto the Amine Reactive Biosensors. The kit contains sufficient reagents for immobilization onto approximately 96 biosensors.

PRINCIPLE

The Amine Reactive Biosensor surface is a biocompatible layer with many available carboxylic acid groups. Treatment of this surface with a mixture of EDC/NHS activates the carboxylic acids toward nucleophilic attack. Subsequent exposure of this activated surface to a protein that is at a pH slightly below its pI, will result in an amide bond formation between a primary amine of the protein and the carboxylate of the surface.

KIT CONTENTS

Amine Coupling Reagent Kit (Part No. 18-5017) is sufficient for 96 immobilizations onto Amine Reactive Biosensors and contains:

	Reagent	Amount provided	Storage
1	EDC [N-(3-Dimethyl aminopropyl)-N'-ethylcarbodiimide]	1009 mg	Store dry reagent desiccated at -20°C
2	NHS [N-hydroxy succinimide ester]	152 mg	Store dry reagent at room temperature
3	1M ethanolamine, pH 8.5	20 mL	Store at 2-8°C
4	100 mM MES, pH 4.0	60 mL	
5	100 mM MES, pH 5.0	60 mL	
6	100 mM MES, pH 6.0	60 mL	
7	Phosphate buffered saline (PBS)	40 mL	

ADDITIONAL MATERIALS REQUIRED

The following additional materials are required:

- 96-well, black, flat bottom, polypropylene microplates (Greiner Bio-one # 655209)
- Distilled, deionized water for solublizing the EDC and NHS
- Purified protein stock (>125ug/mL) of the ligand to be immobilized. Ligand should be free of carrier protein in an amine free buffer.
- Protein of interest (analyte) which binds to ligand
- Octet Instrument and Software version 3.0 or higher

TECHNIQUES FOR OPTIMAL PERFORMANCE

- Equilibrate all reagents and samples to room temperature prior to preparation.
- Avoid contamination of the MES and PBS buffers by decanting the required amounts into an appropriate vessel.
- For frozen samples, thaw and mix thoroughly prior to use.
- Hydration of the biosensors is required prior to assaying on the Octet System.
- A minimum of 200 µL/well is required for samples and the sensor hydration solution.
- Ensure that the Octet instrument is turned on and the lamp is warmed up to room temperature for at least 40 minutes prior to starting the assay.
- Set the sample plate temperature. In the Octet Software select: File ➔ Experiment ➔ Set plate temperature. Enter the desired temperature between room temperature to 40°C. ForteBio recommends assaying at 30°C.

REAGENT PREPARATION AND STORAGE

Upon receipt of kit, separate and store the reagents at the recommended storage conditions.

Prior to first use of the kit, prepare and aliquot EDC and NHS.

EDC stock preparation -

1. Add 13.2 mLs of distilled, deionized water to the vial containing the EDC.
2. Mix to thoroughly solubilize the reagent.
3. Aliquot 1 mL into labeled 2 mL eppendorf tubes (~12 X 1mL aliquots total).
4. Freeze and store aliquots at -20C.

NHS stock preparation -

1. Add 13.2 mLs of distilled, deionized water to the vial containing the NHS.
2. Mix to thoroughly solubilize the reagent.
3. Aliquot 1 mL into labeled 2 mL eppendorf tubes (~12 X 1mL aliquots total).
4. Freeze and store aliquots at -20C.

USING THE KIT FOR IMMOBILIZATION

1. Allow MES, ethanolamine and PBS to come to room temperature prior to use.
2. Thaw 1 aliquot (1 mL) of EDC and 1 aliquot (1 mL) of NHS. Combine to make 2mL of EDC/NHS reagent. This is sufficient reagent for 8 biosensors.

   Note: It is recommended to use the EDC/NHS mixture within one hour.
3. **Ligand preparation -** Dilute the ligand to be immobilized in 100 mM MES at the appropriate pH. Follow the guidelines below for selecting the appropriate pH of MES buffer.

   Note: Some proteins are not stable for long periods of time at these pHs. It is safest to dilute the proteins into the buffer immediately prior to use.

   a. If the pI of the protein ligand is known, dilute the ligand into the MES buffer with the pH closest to, but below, the pI of the protein.

   b. If the pH needed is between the MES buffers provided, refer to **Table 1** for buffer formulation. Volumes in the table make 10 mLs of buffer at the desired pH.

   c. If the pI of the protein is unknown it may be necessary to test the immobilization of the ligand at all three pHs provided (pH 4.0, 5.0, 6.0).

Table 1: Buffer formulation for creation of 10 mL of 100 mM MES at the pH indicated.

Target pH	Volume of 100 mM MES pH 4.0 (mL)	Volume of 100 mM MES pH 5.0 (mL)	Volume of 100 mM MES pH 6.0 (mL)
4.1	9.45	0.55	----
4.2	9.2	0.8	----
4.3	8.7	1.3	----
4.4	8.1	1.9	----
4.5	7.3	2.7	----
4.6	5.4	4.6	----
4.7	4.9	5.1	----
4.8	4.0	6.0	----
4.9	3.3	6.7	----
5.1	----	9.4	0.6
5.2	----	8.9	1.1
5.3	----	8.2	1.8
5.4	----	7.5	2.5
5.5	----	6.2	3.8
5.6	----	5.1	4.9
5.7	----	3.9	6.1
5.8	----	2.6	7.4
5.9	----	1.2	8.8

4. **Sample plate preparation -** Transfer 200 µL of each assay reagent into the 96-well black, flat bottom, polypropylene microplate. Use the plate map in shown **Table 2** in the *Representative Data* section below as guidance.
5. **Hydration plate preparation -** Gently remove the green biosensor rack from the biosensor assembly and place on the bench. Place a 96-well plate securely in the blue biosensor tray holder. Transfer 200 µL of the 100 mM MES buffer into each well in the microplate that matches the number and location of the sensors being used.
6. Set up the Octet Instrument and run the assay according to the Amine Reactive Biosensor package insert.
7. In some cases, the immobilization may need to be further optimized to achieve the desired signal. This can be done by changing protein concentrations, optimizing EDC/NHS concentrations and exploring pH of immobilization. Please contact support@fortebio.com for additional information and guidelines.

REPRESENTATIVE DATA

In the following example recombinant human TNFα was immobilized to the Amine Reactive biosensors. The EDC/NHS mixture was prepared according to the kit instructions. In this example the TNFα was immobilized onto the biosensor surface at different densities. To achieve this goal the TNFα was immobilized at the concentrations indicated in Table 2 in100mM MES, pH 5.0. The 100 mM MES buffer, EDC/NHS, 1M ethanolamine and PBS are included in the Reagent Kit. The TNFα was purchased from eBiosciences (C/N 14-8329-86) and the anti-TNFα antibody was purchased from BD Pharmingen (C/N 552467).

Table 2: Example of the sample plate lay out.

	1	2	3	4	5	6
A	100mM MES pH 5.0	EDC/ NHS	12.5µg/mL TNFα, 100mM MES pH 5.0	1M ethanola mine	PBS	40nM anti-TNFα, PBS
B	100mM MES pH 5.0	EDC/ NHS	12.5µg/mL TNFα, 100mM MES pH 5.0	1M ethanola mine	PBS	40nM anti-TNFα, PBS
C	100mM MES pH 5.0	EDC/ NHS	6.25µg/mL TNFα, 100mM MES pH 5.0	1M ethanola mine	PBS	40nM anti-TNFα, PBS
D	100mM MES pH 5.0	EDC/ NHS	6.25µg/mL TNFα, 100mM MES pH 5.0	1M ethanola mine	PBS	40nM anti-TNFα, PBS
E	100mM MES pH 5.0	EDC/ NHS	3.13µg/mL TNFα, 100mM MES pH 5.0	1M ethanola mine	PBS	40nM anti-TNFα, PBS
F	100mM MES pH 5.0	EDC/ NHS	3.13µg/mL TNFα, 100mM MES pH 5.0	1M ethanola mine	PBS	40nM anti-TNFα, PBS
G	100mM MES pH 5.0	EDC/ NHS	1.25µg/mL TNFα, 100mM MES pH 5.0	1M ethanola mine	PBS	40nM anti-TNFα, PBS
H	100mM MES pH 5.0	EDC/ NHS	1.25µg/mL TNFα, 100mM MES pH 5.0	1M ethanola mine	PBS	40nM anti-TNFα, PBS

Eight Amine Reactive Biosensors were hydrated in 100mM MES pH 5.0 for 5 minutes. The method file listed in **Table 3** was programmed into the Octet Instrument and the assay was run according to the product insert.

Table 3: Assay Method File

Step	Reagent	Time (sec)	Flow (rpm)	Step type
1	100 mM MES pH 5	120	1000	Baseline
2	EDC/NHS	300	1000	Activation
3	TNFa	900	1000	Loading
4	1M Ethanolamine pH 8.5	300	1000	Quench
5	PBS, pH 7.4	300	1000	Baseline
6	Anti-TNFa, 40 nM, PBS	900	1000	Association
7	PBS, pH 7.4	1000	1000	Dissociation

The immobilization and subsequent binding of the antibody were monitored in real time. **Figure 1** shows the Real Time Binding Chart for the assay. From this chart the duplicates of the four different loading densities of TNFα can easily be identified. This is an example of using different immobilization concentrations to achieve different surface densities.

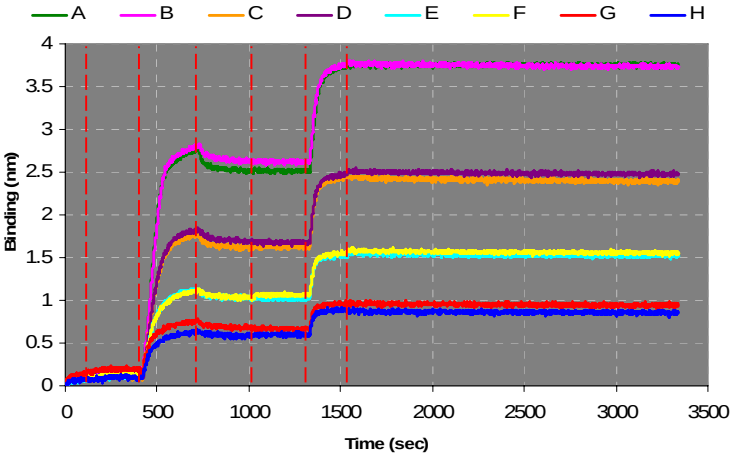

Figure 1: Real Time Binding Chart. Biosensor A (green) and biosensor B (pink) loaded at 12.5 µg/mL of TNFα; Biosensor C (orange) and biosensor D (purple) loaded at 6.25 µg/mL of TNFα; Biosensor E (light blue) and biosensor F (yellow) loaded at 3.13 µg/mL of TNFα; Biosensor G (red) and biosensor H (blue), loaded at 1.25 µg/mL of TNFα.

Data was processed with the Octet User software and the full dissociation option. The calculated kinetic parameters for each sensor are shown in **Table 4**.

Table 4: Kinetic parameters calculated from assay data

Sensor	kdis [1/s]	kassoc [1/Ms]	K_D [M]
A	1.65E-05	8.86E+05	1.86E-11
B	1.69E-05	7.22E+05	2.34E-11
C	2.64E-05	9.75E+05	2.71E-11
D	1.91E-05	9.69E+05	1.97E-11
E	2.87E-05	1.30E+06	2.21E-11
F	2.98E-05	1.21E+06	2.46E-11
G	3.69E-05	1.45E+06	2.54E-11
H	3.44E-05	1.55E+06	2.22E-11

The resulting K_D was consistent across all biosensors regardless of the immobilized surface density of the TNFα.

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